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Short Communication

Clonal Human (hNT) Neuron Grafts for Stroke Therapy

Neuropathology in a Patient 27 Months after Implantation

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Although grafted cells may be promising therapy for stroke, survival of implanted neural cells in the brains of stroke patients has never been documented. Human NT2N (hNT) neurons derived from the NTera2 (NT2) teratocarcinoma cell line were shown to remain postmitotic, retain a neuronal phenotype, survive >1 year in host rodent brains and ameliorate motor and cognitive impairments in animal models of ischemic stroke. Here we report the first postmortem brain findings of a phase I clinical stroke trial patient implanted with human hNT neurons adjacent to a lacunar infarct 27 months after surgery. Neurofilament immunoreactive neurons were identified in the graft site, fluorescent *in situ* hybridization revealed polyploidy in groups of cells at this site just like polyploid hNT neurons *in vitro*, and there was no evidence of a neoplasm. These findings indicate that implanted hNT neurons survive for >2 years in the human brain without deleterious effects. *(Am J Pathol 2002, 160:1201–1206)*

Stroke affects millions of people worldwide with >500,000 new patients per year in the United States. Although less than a third of strokes are fatal,[1] ~60% of patients show significant residual impairments,[2] and the prevalence of stroke-related morbidity will increase as the population ages because there are no therapies that reverse these effects. However, implantation of neural cells in the stroke penumbra may have therapeutic benefits by re-establishing neuronal circuits or enhancing functions of residual penumbral neurons as suggested by some[3–6] but not all[7] studies of stroke in animal models. NT2N (hNT) neurons are derived from a clonal human teratocarcinoma cell line (NTera-2 or NT2), and NT2 cells acquire a permanent postmitotic neuronal phenotype *in vitro* after retinoic acid treatment.[8–11] Cultured and grafted hNT cells exhibit a neuronal phenotype,[11–13] including the formation of functional synapses *in vivo*[14] as well as secretion of neurotransmitters (GABA, dopamine),[15–17] and these neurons are model systems for human neurobiology research.[10–24] In addition, grafted hNT neurons have been used for stroke therapy in rodents,[3,4,6] and a phase I clinical trial of grafted hNT neurons (produced by Layton Bioscience, Inc., Sunnyvale, CA and known as LBS-neurons for human use) for stroke therapy has been completed.[25,26] Here we describe grafted hNT neurons in one of these trial patients.

Materials and Methods

Details of the phase I protocol for implantation of hNT cells in stroke patients have been described.[25,26] Briefly, Leksell model G stereotaxic coordinate frame (Elekta Instruments, Atlanta, GA) was applied to the head under local anesthesia and mild sedation. Computerized to-

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mography was performed for stereotaxic targeting to the stroke site. The cells were prepared in the Cellular Products Laboratory of the University of Pittsburgh Cancer Institute and viable hNT cells were implanted after the dura was opened and a stabilizing probe was inserted to a point 4-cm proximal to the final target. The cells were aspirated into a 250-μl syringe, the cannula was filled with the cell suspension, and a 20-μl volume of cells was injected slowly at the first target site. The instrument was then withdrawn to the second and third sites for subsequent implants. Immunosuppression was begun 1 week before surgery and discontinued 8 weeks after surgery.

The focus of the current study was a 71-year-old man with fixed motor deficits (marked left facial weakness, slight proximal movement of the left arm and fingers, mild leg weakness, but no dysarthria or aphasia) because of lacunar stroke of the right putamen. He received stereotaxic implantation of 2×10^6 hNT neurons 34 months after infarction adjacent to the infarct in a phase I clinical trial (Figure 1A). This patient died of a presumptive myocardial infarction 27 months after implantation, but unlike 6 of the 12 patients in the trial who showed motor improvements,[25] this patient demonstrated no motor recovery. The patient was embalmed, the brain removed and then transported to the University of Pennsylvania Hospital for neuropathological examination. Blocks of tissue were obtained from the entire lacune and graft site as well as multiple areas of isocortex, allocortex, brainstem, and cerebellum after further fixation in 10% neutral buffered formalin. Near serial paraffin-embedded sections were cut for histochemistry, including hematoxylin and eosin (H&E), Bodian, and Luxol Fast Blue/Nissl staining, as well as immunohistochemistry with antibodies to glial fibrillary acidic protein, neurofilament (NF) proteins (RMD020), and Ki-67 (MIB-1) as described.[4,10,14]

Fluorescent *in situ* hybridization (FISH) was performed on 4-μm-thick paraffin-embedded sections using Vysis' LSI probes (Vysis, Downer's Grove, IL) according to the manufacturer's recommendations with two different sets of probes. To visualize chromosome 21 alone, Vysis' LSI 21 SpectrumOrange (loci D21S259, D21S341, D21S342, region 21q22.13-q22.2; product no. 32-190002) was used. Vysis' MultiVysion PGT (product no. 32-131080) was used to visualize both chromosome 13 (13q14; SpectrumRed) and chromosome 21 (21q22.13-q22.2; SpectrumGreen).

Tissues studied by FISH included the brain of the current patient (including the graft site wherein neurons were identified by immunohistochemistry), a control human brain, and the graft site in rat brains previously injected with hNT neurons. Cells with fluorescent signals were enumerated in at least 50 nonoverlapping nuclei in each case using a Nikon fluorescent microscope equipped with a triple band-pass filter set. The numbers of signals per nucleus were counted for each cell. Because hNT neurons required FISH and oil immersion fluorescence microscopy for identification, stereology could not be used to count the total hNT neurons in the graft site.

Results

On neuropathological examination of the 1246 g brain, macroscopic abnormalities were an $\sim 3 \times 1.5 \times 2$-cm resolved right putamen lacune encroaching on the internal capsule and an ~ 1-cm^3 gliotic area medial to the infarct consistent with the site of the grafted hNT neurons (Figure 1, A and B). Although spinal cord was not available for study, corticospinal tract degeneration was noted to extend from the internal capsule through brainstem (Figure 1C). Histological examination demonstrated extensive gliosis with numerous glial fibrillary acidic protein-immunoreactive astrocytes and some hemosiderin-laden macrophages surrounding the infarct. Bodian and Luxol Fast Blue myelin stains revealed extensive loss of myelinated axons in the lacune and throughout the ipsilateral corticospinal tract (Figure 1D). No tumor was identified anywhere in the brain, and a monoclonal antibody to Ki-67, a protein expressed in cycling cells, immunolabeled $\ll 1\%$ of cells (data not shown), consistent with the absence of a neoplasm. There was no evidence of additional infarcts, vasculopathy, inflammatory/infectious disorders, neurodegenerative disease, congenital anomalies, and so forth.

Histochemical stains demonstrated a population of neurons at the graft site (Figure 1E), and many were NF protein immunoreactive consistent with grafted hNT neurons in experimental animals (Figure 1F). However, implanted hNT neurons could not be identified unequivocally by immunohistochemistry in human brain, although hNT neurons can be identified in rodent brain with human species-specific antibodies to neuronal proteins.[4,10,20–23] To overcome this problem and identify neurons in the graft site as hNT neurons, we exploited the known stable polyploidy of hNT neurons in FISH studies of sections through the graft site containing NF-positive neurons.[21] Because hNT neurons have been shown to be polyploid for chromosome 21, but not for chromosome 13, we used FISH with DNA probes designed for use on formalin-fixed paraffin-embedded tissue. Accordingly, we conducted FISH to probe sections of the graft site in the patient's brain, a control human brain, and the graft site in a rat brain previously injected with hNT neurons. A single chromosome 21-specific probe detected multiple signals in nuclei within the previously identified hNT neuron graft site of the rat brain (Figure 2A). Similarly, polyploidy for chromosome 21 was observed at the hNT neuron injection site in the current patient (Figure 2B). Using a cocktail of FISH probes for multiple chromosomes, we evaluated chromosome numbers in nuclei of neurons at the hNT graft site and demonstrated polyploidy in chromosome 21, but not chromosome 13, at the hNT neuron implantation site in the current patient (Figure 2; C to F and Figure 3). Thus, the presence of cells with a neuronal phenotype and distinctive chromosomal features of hNT neurons is consistent with survival of a population of grafted hNT neurons in the brain of this patient 27 months after implantation.

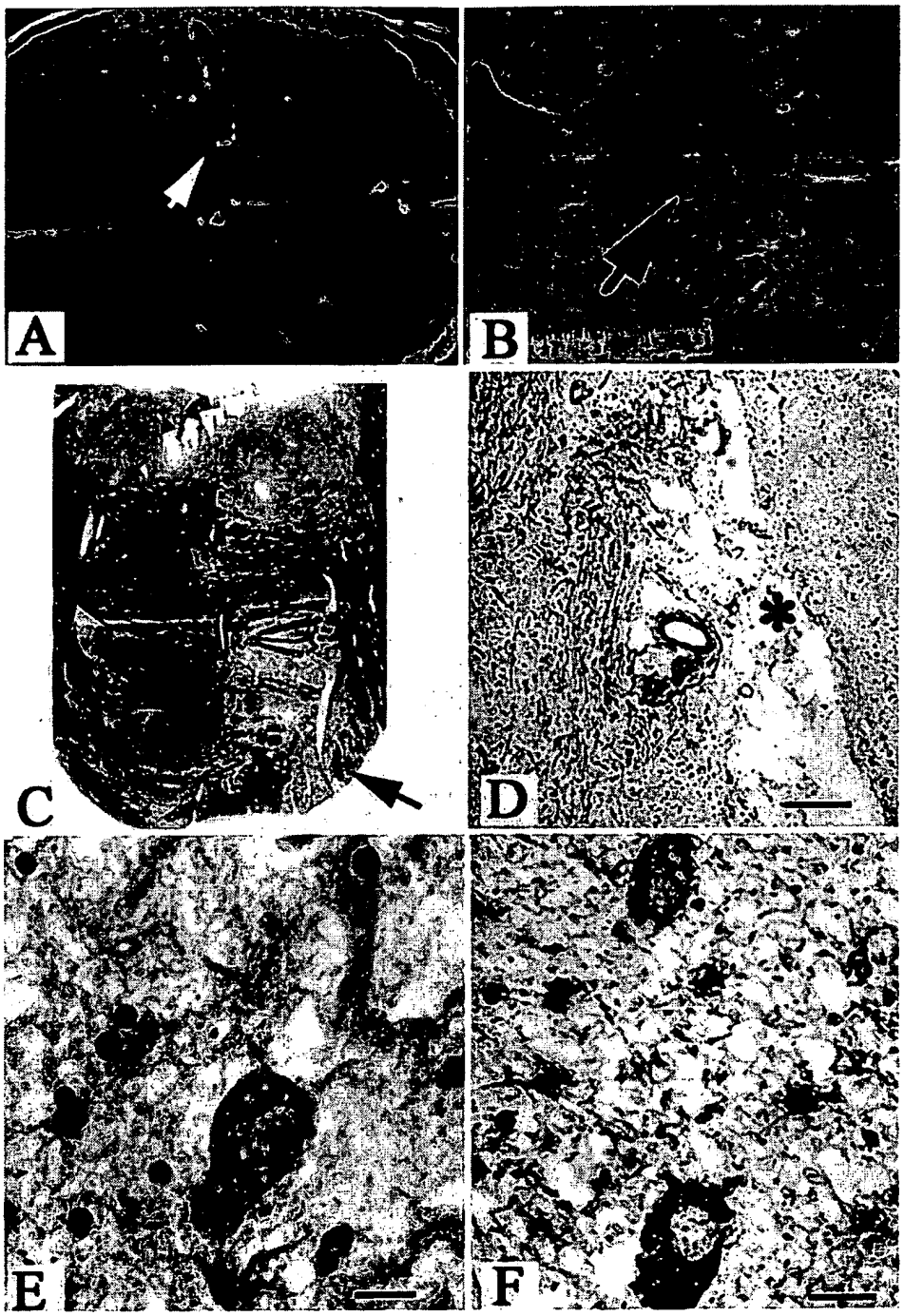

Figure 1. Radiographical and neuropathological features of lacunar infarct and hNT graft site. **A:** Preoperative CT scan of the patient's brain in horizontal plane shows the location of the putamenal infarct (**arrow**) and the target site of the hNT2 graft (+). **B:** Gross photograph of brain (cut in the coronal plane) shows the infarct (**green arrows**) as well as the site of the grafted hNT neurons medial to the infarct (**blue arrow**). **C:** Low-power photomicrograph of a Luxol Fast Blue-stained section of pons showing myelin loss indicative of corticospinal tract degeneration on the right side (**arrow**) ipsilateral to the infarct. **D:** Histopathology of the lacune (**asterisk**) with disrupted axons (**left**) and gliosis (**right**) as revealed in a Luxol Fast Blue-stained section (scale bar, 1 mm). **E:** H&E-stained section shows a neuron and gliosis within the graft site (scale bar, 20 μm). **F:** Immunohistochemistry with an anti-NF protein antibody (RMD020) shows two NF-positive neurons surrounded by gliosis within the graft site (scale bar, 20 μm).

Discussion

The findings reported here confirm that grafted hNT neurons do not revert to a neoplasm, consistent with previous studies in experimental animals for >1 year[12–14] and they suggest that a population of transplanted hNT neurons survived in the brain of this patient for 27 months after implantation. Although mature neurons duplicate chromosomes before cell death in Alzheimer's disease,[27] and fibroblasts from Alzheimer's disease patients can be trisomic for chromosome 21,[28] which could confound inter-

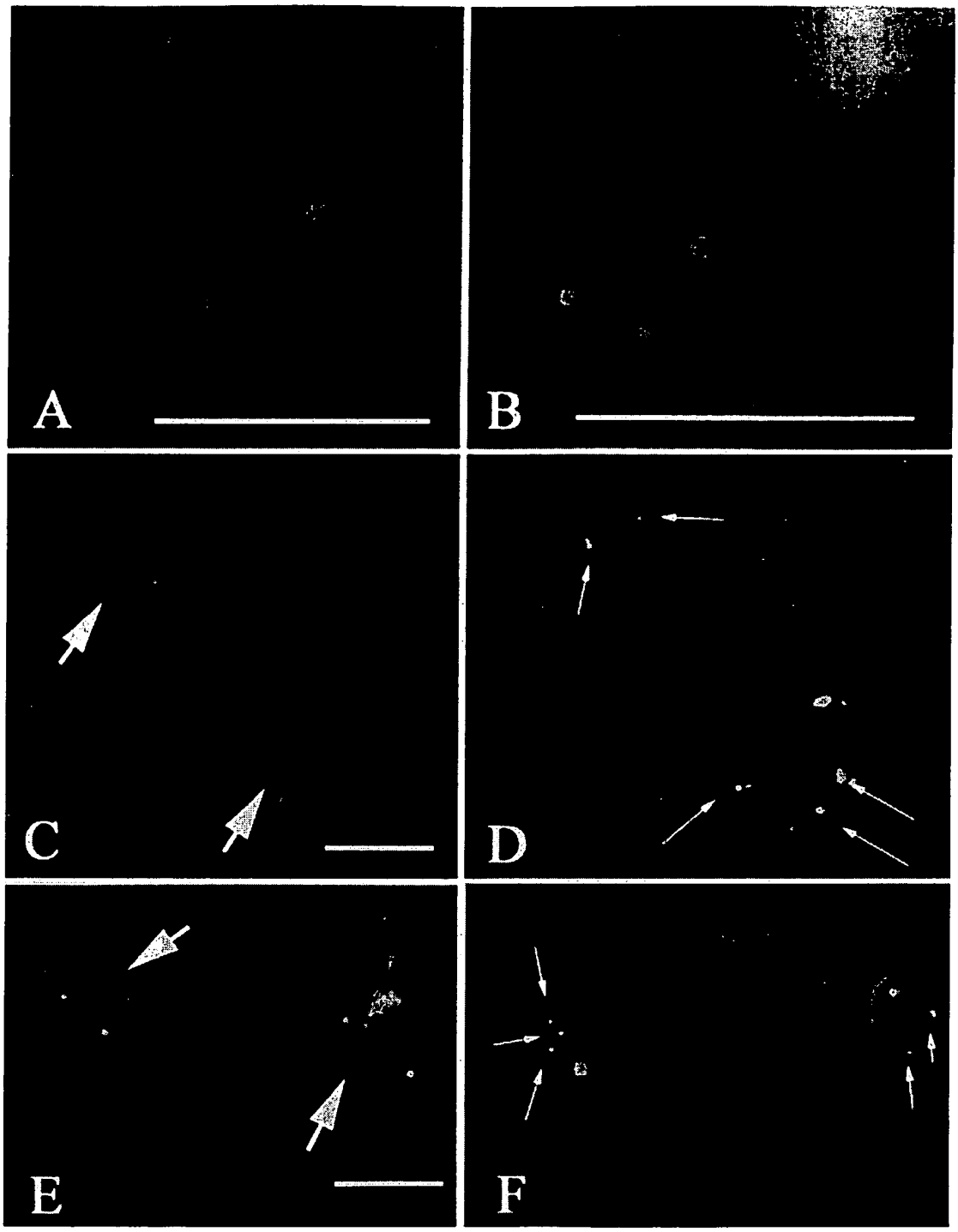

Figure 2. FISH signals within neuronal nuclei in hNT graft site. **A:** A section through the graft site in the brain of rat subjected to ischemic stroke was probed by FISH as a positive control to demonstrate hNT neurons polyploid for chromosome 21 as evidenced by three red fluorescent FISH signals in the nucleus of the neuron shown at high magnification here (scale bar, 20 μm). **B:** Using the same chromosome 21 probe as in **A**, FISH shows polyploid nuclei (three small red fluorescent FISH signals at **bottom left**) in a neuron at the graft site of the current patient in sections adjacent to those in Figure 1, E and F (scale bar, 20 μm). The fluorescence at the **top right** is lipofuscin. **C–F:** FISH staining of sections adjacent to those in Figure 1, E and F, shows more than two signals for chromosome 21 but two signals for chromosome 13 in neurons within the implantation site (scale bars, 40 μm). **C** and **E** (**left**) show neurons viewed with a rhodamine filter to visualize FISH signals corresponding to chromosome 13 in red (**white arrows**) whereas **D** and **F** (**right**) show neurons viewed with a FITC filter to demonstrate FISH signals corresponding to chromosome 21 in green (**yellow arrows**). The **blue arrow** in **E** indicates a large autofluorescent lipofuscin accumulation.

pretation of our FISH data here, the current patient did not have Alzheimer's disease and the control brain did not have polyploid neurons (Figure 3).

Although there have been a few previous studies describing the neuropathology of human brain transplant efforts, these implants were performed for therapy of

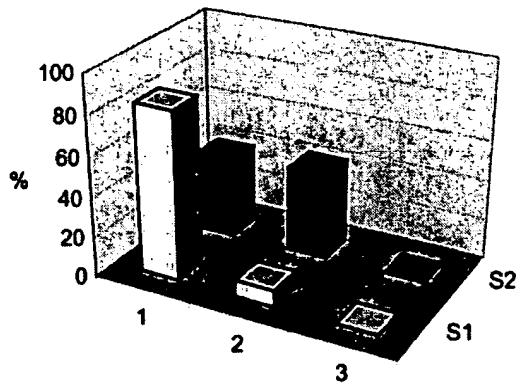

Figure 3. A comparison of the relative abundance of polyploid nuclei in sections from a control human brain (S1 in yellow) and the hNT graft site in the brain of the present case (S2 in black) examined by FISH with probes for chromosomes 13 and 21 using the semiquantitative procedures described in the text. The largest percentage of cells in both brains contain nuclei with two or fewer FISH signals (**row 1**), whereas a negligible percentage of cells contain nuclei with more than two FISH signals for both chromosomes 13 and 21 (**row 3**). However, relative to the control brain, the graft site in the brain of the stroke patient shows a high percentage of cells containing nuclei with more than two FISH signals predominantly for chromosome 21 (**row 2**).

neurodegenerative disease, and used embryonic human neural cells.[12,14,29,30] In contrast, hNT cells are well-studied clonal human neurons with several distinct advantages for use in therapy of human brain diseases. For example, they: 1) do not pose ethical or legal problems because they are not derived from human embryos; 2) are highly uniform unlike cells cultured from living animals; 3) do not harbor known human pathogens or potentially infectious agents present in xenografts; 4) are available in unlimited quantities produced in accordance with general manufacturing procedures for human use; 5) have been extensively characterized *in vitro* and are amenable to genetic engineering;[11–13] and 6) have been transplanted and characterized previously in normal rodents as well as in animal models of stroke, Huntington's disease, Parkinson's disease, and trauma, with encouraging results.[3,4,6,18,22,24]

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